Human CD271-positive melanoma stem cells associated with metastasis establish tumor heterogeneity and long-term growth

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Abstract: Human melanoma is composed of distinct cell types reminiscent of neural crest derivatives and contains multipotent cells that express the neural crest stem cell markers CD271(p75(NTR)) and Sox10. When isolated from solid tumors by using a method that leaves intact cell surface epitopes, CD271-positive, but not CD271-negative, cells formed tumors on transplantation into nude or nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. These tumors fully mirrored the heterogeneity of the parental melanoma and could be passaged more than 5 times. In contrast, in more immunocompromised NOD/SCID/IL2r (null) mice, or in natural killer cell-depleted nude or NOD/SCID mice, both CD271-positive and CD271-negative tumor cell fractions established tumors. However, tumors resulting from either fraction did not phenocopy the parental tumors, and tumors derived from the CD271-negative cell fraction could not be passaged multiple times. Together, our findings identify CD271-positive cells as melanoma stem cells. Our observation that a relatively high frequency of CD271/Sox10-positive cells correlates with higher metastatic potential and worse prognosis further supports that CD271-positive cells within human melanoma represent genuine cancer stem cells. Cancer Res; 71(8); 3098-109. ©2011 AACR.

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Cancer Research

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Title

Human CD271-Positive Melanoma Stem Cells Associated with Metastasis Establish Tumor Heterogeneity and Long-Term Growth

Running Title: Human Melanoma Contains CD271-Positive Melanoma Stem Cells

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Human melanoma is composed of distinct cell types reminiscent of neural crest derivatives and contains multipotent cells that express the neural crest stem cell markers CD271($p75^{NTR}$) and Sox10. When isolated from solid tumors using a method that leaves intact cell surface epitopes, CD271-positive but not CD271-negative cells formed tumors upon transplantation into Nude or NOD/SCID mice. These tumors fully mirrored the heterogeneity of the parental melanoma and could be passaged more than 5 times. In contrast, in more immunocompromized NOD/SCID/IL2rγ^{null} (NSG) mice, or in NK cell-depleted Nude or NOD/SCID mice, both CD271-positive and CD271-negative tumor cell fractions established tumors. However, tumors resulting from either fraction did not phenocopy the parental tumors, and tumors derived from the CD271-negative cell fraction could not be passaged multiple times. Together, our findings identify CD271-positive cells as melanoma stem cells. Our observation that a relatively high frequency of CD271/Sox10-positive cells correlates with higher metastatic potential and worse prognosis further supports that CD271-positive cells within human melanoma represent genuine cancer stem cells.

Introduction

Tumors usually are heterogenic and comprise cells with different capacities to proliferate and differentiate. It has been proposed that this cellular heterogeneity depends on the presence of so-called cancer stem cells, which are defined as cells that can induce de novo tumor formation, self-renew in vivo, and re-establish the cellular composition of the parental tumor (1). Although the cancer stem cell concept is accepted for several types of tumors (2), data for human melanoma, the most aggressive skin cancer, are conflicting (3). For example, melanoma cells exhibiting stem cell properties in vitro displayed increased tumorigenicity as compared to melanoma cells lacking self-renewal potential upon xenotransplantation into immunodeficient Nude or NOD/SCID mice (4). Later studies suggested that the expression of markers such as CD20, CD133, MDR1, are associated with melanoma stem cells (4-6), but a strict correlation between marker expression, self-renewal in vitro and in vivo, multi-lineage differentiation, and high tumorigenicity remains to be established (7). More recently, ABCB5 has been identified as a marker of melanoma-initiating cells capable of self-renewal and differentiation and associated with clinical melanoma progression in human patients (8). Similarly, in fully immunocompromised mouse models, including NOD/SCID/IL2rγ^{null} (NSG) mice, melanoma cells expressing the neurotrophin receptor CD271 (p75^{NTR}) had a higher tumor-initiation capacity than CD271-negative cells, although the negative fraction was also able to generate tumors in these mouse models (9).

In contrast, other recent studies reported a generally high frequency of tumorigenic melanoma cells when NSG mice were used as recipients (10, 11). In addition, these studies did not find a correlation between the capacity to form a tumor and the

expression of previously published markers, including CD271 and ABCB5, thus questioning the concept of cancer stem cells (10, 11).

Because cancer stem cells by definition must be able to reproduce the full heterogeneity of the parental tumor and to grow continuously even after multiple passages, we embarked to compare those aspects for the tumors arising in NOD/SCID, Nude and NSG mice after transplantation of CD271-positive vs. -negative melanoma cell fractions. To this end, the cells were isolated from human melanoma samples applying an enzymatic tumor digestion protocol that does not harm surface epitopes. Using this gentle method of cell fraction preparation, we reveal that CD271-positive melanoma cells meet the definition of melanoma stem cells. Intriguingly, the incidence of such cells in patient biopsies is associated with poor prognosis.

Materials and Methods

Tissue microarray (TMA) analysis

All analyses involving human melanoma tissue were performed in accordance with the ethical committee in canton Zurich. The TMA used here comprised 54 primary melanomas, 141 melanoma metastases and 53 melanoma cell lines and was generated as described (12, 13). Co-expression of CD271 and Sox10 was identified by double immunostaining (Sox10, 1:800, R&D, MBA2864; CD271, 1:50, Miltenyi Biotec, 130-091-883). The staining procedure was done as previously described (12). The frequency of cells that express CD271 plus Sox10 are presented as mean ± standard deviation (SD) and median with interquartile range (IQR). Values were compared using the Mann-Whitney-U test with exact p-values. Tumor-specific survival was analyzed using Kaplan-Meier curves and compared between groups by log rank test. SPSS 15 software (SPSS Inc., Chicago, IL) was used for statistical analyses. Two-tailed p-values less or equal to 0.05 were considered statistically significant.

Mice

BALB/c-Swiss nude (CAnN.Cg-*Foxn1*^{nu}/Crl, Nude) and NOD/SCID/IL2rγ^{null} (NOD.Cg-Prkdcscid Il2rgtm1Wjl/S, NSG) mice were purchased from Charles River (Germany). NOD/SCID (NOD.CB17/JHliHsd-*Prkdc*^{scid}, N/S) mice were obtained from Harlan Laboratories (Switzerland). Mice were housed under standard conditions with free access to water and food. Experiments were performed with male or female mice o 6-10 weeks of age in accordance with the Swiss federal and cantonal laws on animal protection.

Tumor cell isolation and xenotransplantation

All patients enrolled in the study were treated at the Dermatology Department of the University Hospital of Zurich. The study was approved by the ethics committee of canton Zurich and all patients gave informed consent. Immediately after surgical resection, the solid, metastatic lesions were dissociated into single-cell suspensions using HBSS (without Ca^{2+} and Mg^{2+}, Invitrogen) containing collagenase III (1mg/ml, Worthington Bioch.) and dispase (0.5 mg/ml, Roche). Incubation at 37°C for 1h with concurrent mincing allowed complete digestion. In some cases, xenografts and cell lines were dissociated as indicated above, followed by an additional digestion with trypsin (0.05% trypsin-EGTA, Gibco) for 5 min at 37°C, exactly as described (10, 11). The resulting cell suspension was filtered through 40 µm nylon mesh and single cells were harvested. 1 cm^2 tumor xenografts were harvested from euthanized mice and dissociated as described above. 1,000 bulk tumor, in vitro cultured or FACS-sorted cells were resuspended in Matrigel matrix (BD Biosciences) 1:1 diluted with RPMI-1640 (Invitrogen) and 200 µl were injected s.c. in the flank of mice with a 1-ml syringe with a 25-gauge hypodermic needle.

Immunofluorescence

Paraffin embedded, 4-µm formalin fixed tissue sections were deparaffinized in xylene and rehydrated. Following heat-induced epitope retrieval using 10 mM trisodium citrate buffer at pH 6.0 and a Microwave Histoprocessor (Milestone), the sections were incubated over-night at 4°C as described with antibodies for the following markers: Sox10 (1:800, R&D, MAB2864), CD271 (1:200, Alomone lab, ANT-007), MelanA (1:50, Abcam, Ab785), S100P (1:400, DAKO, Z0611), HMB45 (1:100, DAKO,

M0634), MITF (1:100, Acris, DM4625), TuJ1 (1:200, Sigma, T8660), NF (1:200, Sigma, N5264), NSE (1:100, Abcam, Ab53025), Peripherin (1:100, Abcam, ab4666), SMA (1:400, Sigma, A2547) and GFP (1:500, Abcam, Ab290). Sections were subsequently incubated with following secondary antibodies for 1 h at RT: Cy3-conjugated goat anti-mouse (1:500, Jackson ImmunoResearch Laboratories), Cy3-conjugated goat anti-rabbit (1:500, Jackson ImmunoResearch Laboratories), Alexa 488-conjugated goat anti-mouse (1:500, Invitrogen). All slides were counterstained with Hoechst 33342 (Invitrogen). In addition, sections were stained with hematoxilin and eosin (H&E). Stainings on cultured cells were done as described (14).

Quantification of positive cells was performed using ImageJ software (National Institutes of Health). Three images from each biopsy were taken randomly. Images were imported into ImageJ, converted to 8-bit formats, and subjected to automatic threshold. The "Analyze Particles" function was used to count positive cells.

Flow cytometry and FACS-sorting

All steps for flow cytometry were performed in RPMI-1640 supplemented with 10% FCS (Invitrogen), 5% Pen/Strep (Invitrogen) and 2mM EDTA. For sorting or analysis, single cell tumor samples were stained with anti-human CD271 (Miltenyi Biotec, FITC-conjugated: 130-091-917, or APC-conjugated: 130-091-884), anti-human CD44 (1:100, BD Pharmingen, 555477), or anti-ABCB5 antibody (1:200, clone 3C2-1D12; a gift from Markus Frank). Samples were measured with a FACScanto II (BD Biosciences) an analyzed with Diva software (BD Biosciences). Sorting was performed with a FACSAria (BD Biosciences) and the purity of sorted fractions was 95 to 99% of CD271-positive

cells in the CD271-enriched fraction and less than 0.5% of CD271-positive cells in the CD271-depleted fraction.

NK-cell depletion

Mice were injected i.p. with 50 µl polyclonal rabbit anti-asialo GM1 antibodies (Wako Chemicals, cat. 986-10001) in 150 µL PBS every 5 days starting at the day of melanoma cells engraftment, which resulted in a >95% depletion of CD3- DX5+ NK cells for the duration of the experiment as measured by flow cytometry (15).

Sphere culture

Sphere cultures were established as previously described for neural stem cells (16). Briefly, single cells were plated in flasks (Nunc) coated with Poly(2-hydroxyethylmethacrylate) (Poly-Hema, Sigma) at a density of 20,000 viable cells/ml in DMEM-F12 1:1 media (Gibco) containing 1x B-27 supplement (Invitrogen), 20 ng/ml FGF2 (PeproTech), 10 ng/ml EGF (PeproTech), 5% Pen/Strep (Invitrogen). The resulting spheres were collected after 7-10 days by gentle centrifugation (800 rpm), dissociated using PBS containing 2 mM EDTA and replated into Poly-Hema coated culture flasks. To ensure plating of single, viable cells, the dissociated sphere cells were passed through a 40 µm nylon mesh followed by Trypan blue examination. To assess the relative sphere numbers over passage, spheres were grown for 7 days, counted, dissociated and replated under the same conditions.

Melanoma cell lines

Cell lines M990115 and M010817 were previously described (17). The cell line M070302 was established from surplus material from a xenograft (patient 2481) generated in Nude mice. Cell lines were grown in sphere culture condition as describe above.

Transduction of melanoma with GFP-expressing lentivirus

The GFP-expressing lentivirus was produced as described (18). Lentivirus-containing supernatants were collected 48 hours after transfection, filtered through a 0.22 μm membrane and transferred to target cells (see below). We xenografted a fresh human melanoma into Nude mice, processed the xenograft into single cells and cultured them under sphere conditions. Cells were transduced with GFP-lentivirus at the 8th passage. After one week, resulting GFP-tagged cells were checked by FACS and injected s.c. into Nude mice. The GFP-tagged xenografts were collected after 2 months, dissociated into single cells and GFP-positive cells were purified by FACS-sorting.

Generation of GFP-tagged clones

After in vitro transduction, GPF-expressing cells were stained for CD271 and single-cell sorting was performed on a BD FACSAria sorting system. Fibronectin (Sigma)–coated 96-wells flat bottom tissue culture plates (Nunc) were used for 1 cell per well sorting into 200 μL of medium (RPMI-1640 supplemented with 10% FCS, 2mM glutamine, 5% Pen/Strep) per well. Each well was checked with a fluorescence microscope and only wells containing a single cell were processed further. After 2 weeks in culture, clones were stained for CD271 expression and three selected clones were processed as follows:

The clone was divided into two aliquots, one aliquot was cultured for 4 h as described above, fixed and stained for different markers (see above), the other aliquot was xenografted into Nude and NSG mice.

Results

Xenotransplantation into highly immunocompromised mouse models fails to phenocopy the cellular heterogeneity of parental human melanoma samples

As melanoma derives from the neural crest cell lineage, we expect putative melanoma stem cells to exhibit features of neural crest stem cells (NCSCs) and individual tumors to comprise cells expressing the NCSC markers CD271 and Sox10 (19, 20) as well as cells with features of different neural crest derivatives. To examine the cellular heterogeneity of a given patient's tumor, we analyzed 19 independent melanoma metastases (Supplementary Table S1) for the expression of 11 NCSC, melanocytic, neural, and mesenchymal markers and found that the majority of the samples were positive for most or all of these markers (Fig. 1A-B; Supplementary Fig. S1).

To investigate mechanisms of human melanoma formation and propagation, it is imperative to use a model that faithfully re-produces the parental phenotype, which we will call phenocopy hereafter. Melanoma cell xenotransplantation into different models of immunodeficient mice, including highly immunocompromised NSG mice, previously led to conflicting results (3, 7). To address which mouse model(s) for melanoma formation might best re-capitulate the cellular composition of parental tumors, 1000 cells from the bulk of various patient samples were injected subcutaneously into Nude, NOD/SCID, and NSG mice, respectively, and sections of the resulting xenografts were stained for the expression of 11 markers. The transplanted bulk melanoma cells gave rise to tumors in all cases. Importantly, 100% of all xenografts in Nude and NOD/SCID mice were exact phenocopies of the parental tumors with respect to the presence of all markers tested (Fig. 1; Supplementary Fig. S2; Table 1). In contrast, all xenografts in

NSG mice (n = 7) differed from the parental tumors and lacked cells expressing one or more of the following markers: CD271, MITF, S100P or neuronal markers (Fig. 1; Supplementary Fig. S2; Table 1). Quantification of cells expressing a given marker confirmed the consistency of CD271/Sox10, S100P, MITF, expression between Nude and NOD/SCID xenografts and the respective parental tumors, and the discrepancies in marker expression between NSG xenografts and original patient tumors (Fig. 1C).

Because the major difference between NSG mice and Nude or NOD/SCID mice is the absence of NK cells in NSG mice, we addressed whether NK cells influence the phenotype of xenografted tumors by depleting NK cells from Nude and NOD/SCID mice (15). Intriguingly, xenotransplanted melanoma cells did not phenocopy the parental tumor in NK-depleted NOD/SCID mice (Fig. 1A-C; Supplementary Fig. S3; Table 1). Thus, the level of immunocompetence of the xenotransplant recipient crucially affects the capacity of human melanoma cells to form tumors resembling the respective patient melanoma.

CD271-positive melanoma cells are multipotent and able to establish the heterogeneity of the parental tumor

It has recently been debated whether CD271-positive melanoma cells isolated from patient tumors possess an increased tumorigenic capacity in fully immunocompromised mice as compared to CD271-negative cells (9-11). Notably, in these conflicting reports and other studies different enzymatic procedures have been used to prepare cell fractions from solid melanoma samples: Tumors have been mechanically dissociated followed by enzymatic digestion either with collagenase (8), a mix of collagenase and dispase (9), or

collagenase and trypsin (10, 11, 21). Therefore, we addressed whether these different tumor digestion protocols might affect detectability of surface markers used to directly isolate putative tumorigenic melanoma cell populations. Strikingly, the percentage of retrievable CD271- and ABCB5-positive cells was consistently reduced in conditions that include a trypsin incubation step (11), whereas CD44 was not affected (Supplementary Fig. S4). These results demonstrate that a strong proteolytic activity can selectively damage surface protein epitopes and thus lead to reduced detectability by the corresponding antibodies. This unavoidably results in underestimation of surface marker expression in human melanoma cells and in negative fractions being contaminated by cells actually expressing the selected marker. For this reason, in the present study human biopsies and xenografts were dissociated in the absence of trypsin activity.

Using this gentle protocol for tumor dissociation and cell isolation, FACS-sorted CD271-positive melanoma cells from primary xenografts consistently promoted tumor formation in Nude and NOD/SCID mice, while the CD271-negative cell fraction never gave rise to tumors (Fig. 2A) (32 transplantations representing 7 distinct patient tumors). In contrast, we did not detect differences in tumor initiation in NSG mice between CD271-positive and CD271-negative cells from primary xenografts (12 transplantations each, representing 3 distinct patient tumors) (Fig. 2A). Strikingly, in NK-depleted Nude or NOD/SCID mice both CD271-positive and CD271-negative cells initiated tumor formation. Hence, the tumorigenic potential of melanoma cell subpopulations is influenced by the presence of NK cells in xenograft recipients.

Because a cancer stem cell by definition must display the differentiation capacity reflecting its parental tumor, we investigated the heterogeneity of different successfully xenografted tumors in NOD/SCID, Nude, and NSG mice and of the corresponding parental tumor. The cellular composition of all Nude and NOD/SCID xenografts derived from CD271-positive cells (tumors representing 7 distinct patients) was analogous to the corresponding parental tumors (Fig. 2B; Supplementary Fig. S5). In contrast, in NSG mice neither CD271-positive nor CD271-negative cells were able to fully phenocopy the cellular heterogeneity of the corresponding parental tumors with respect to all 11 markers tested (Fig. 2B; Supplementary Fig. S5; Table 2). Of importance, both the CD271-positive and -negative cell fractions failed to consistently generate xenografts expressing crucial melanoma markers such as MITF and S100P or neuronal markers present in the parental tumors. Moreover, none of the tumors produced by CD271-negative cells in NSG mice comprised CD271-positive cells, despite the presence of such cells in the parental tumors (Fig. 2B; Supplementary Fig. S5; Table 2) (12 transplantations representing 3 patients' tumors). In summary, faithful phenocopies of original patient tumors were only achieved upon transplantation of CD271-positive melanoma cells into Nude and NOD/SCID mice, but not into NSG mice.

The fact that CD271-positive cells generate the full heterogeneity of human melanoma upon xenotransplantation into NOD/SCID or Nude mice suggests that these cells are multipotent. To specifically address this issue, we infected melanoma cells from a xenograft of patient sample 2481 with a GFP-expressing lentivirus, followed by FACS of GFP/CD271-double positive cells and plating those cells at clonal density. While 19 ± 4% of the CD271-positive cells gave rise to clones consisting of more than 10 cells after

2 weeks in culture, such clones were not observed in the CD271-negative cell fraction (Fig. 2C). Three clones derived from GFP/CD271-double positive founder cells were selected and subjected to differentiation assays in cell culture, revealing the emergence of multiple cells types, such as cells expressing CD271/Sox10, neuronal and melanocytic markers, and smooth muscle actin (SMA) (Fig. 2D). Thus, the CD271-positive melanoma cell fraction contains a population that is clonogenic and multipotent in cell culture, similar to NCSCs.

To address the multipotency of CD271-positive melanoma cells in vivo, cells from the three selected clones were grafted subcutaneously into Nude and NSG mice, and the xenografts were analyzed for expression of the 11 markers as described before. In Nude mice, the three clones were able to establish a cellular hierarchy with all 11 markers present in the xenografts. In particular, 10.2 ± 2.0 % of the xenograft cells derived from a CD271-positive cell expressed CD271. Moreover, although most smooth muscle cells present in the xenografts were host derived, all Nude xenografts comprised cells double positive for SMA and GFP, revealing that some smooth muscle cells in the tumor originated from CD271-positive melanoma cells. In contrast, neither the NCSC markers CD271/Sox10 nor S100P, MITF, and neuronal traits were consistently expressed in xenografts derived from clones transplanted into NSG mice. Moreover, we did not find any GFP/SMA-double positive cells in the NSG grafts, suggesting that the NSG host environment either suppresses the in vivo multipotency of CD271-positive cells or supports the growth of melanoma cells with restricted developmental capacities (Fig. 2D; Supplementary Fig. S6).

CD271-positive melanoma cells have self-renewal capacity and sustain long-term tumor growth in vivo

To address whether CD271-positive cells have self-renewal capacity, in addition to being multipotent (Fig. 2B-D), we performed sphere assays under conditions that are known to support sphere formation and self-renewal of normal skin-derived NCSCs (16). CD271-positive cells isolated from fresh melanoma tissue or from melanoma cell lines readily generated spheres that contained CD271/Sox10-positive cells and that could be propagated by serial passaging, whereas the few spheroid aggregates generated by the CD271-depleted melanoma cell fraction displayed a reduced passaging capacity (Fig. 3A-B). Determining the ratio between numbers of secondary over primary and quinternary over quaternary spheres showed that CD271-positive but not CD271-negative cells have an extensive self-renewal potential and expand their stem cell activity over time (Fig. 3B).

To confirm the self-renewal potential of CD271-positive melanoma cells in vivo, we isolated CD271-positive and CD271-negative cells from primary xenografts generated by bulk tumor cells in Nude mice and re-transplanted these cell fractions. We found that only CD271-positive cells were able to generate secondary tumors in Nude mice (Fig. 3C-D; Supplementary Fig. S7, "1st passage"; n = 4 for each biopsy). Likewise, CD271-positive cells re-isolated from secondary xenografts consistently generated tertiary tumors, unlike CD271-negative cells (Fig. 3C-D; Supplementary Fig. S7, "2nd passage"). To test whether the cellular heterogeneity originating from CD271-positive cells is a stable trait in vivo, we analyzed first and second passage xenografts by immunofluorescence. Intriguingly, not only the cellular heterogeneity with respect to

neural crest-specific differentiation markers (Fig. 3D; Supplementary Fig. S7), but also the frequency of CD271-positive cells was maintained in xenografts directly generated from unsegregated patients' melanoma cells and in tumors obtained by serial in vivo passaging of CD271-positive cells (Fig. 3C; % of CD271-positive cells in primary xenograft, 1st and 2nd passage, respectively: 10 ± 1%, 11 ± 3%, 8 ± 2%; n = 4). Hence, CD271-positive melanoma stem cells faithfully phenocopy the parental tumor in Nude mice, even after multiple passages in vivo.

Our data suggest that the presence of CD271-expressing melanoma stem cells is a prerequisite for long-term tumor propagation. Therefore, although CD271-negative melanoma cells can generate tumors in NSG, NK-depleted NOD/SCID or NK-depleted Nude mice (Fig. 2) (9, 10), we expected that such tumors cannot be maintained in vivo over a prolonged time period. To test this, we serially passaged tumors generated in NSG mice from CD271-positive or CD271-negative cells, using tumors produced by CD271-positive melanoma cells in Nude mice as positive control. In both Nude and NSG mice, tumors originating from CD271-positive cells allowed propagation in vivo over 5 passages, in accordance with the above described in vivo self-renewal capacity of CD271-positive cells in Nude mice (Fig. 4). In striking contrast, tumors derived from CD271-negative cells in NSG mice exhausted with time and could only be propagated in vivo for three to four passages (Fig. 4). Quantification of CD271-expressing cells in consecutively produced NSG xenografts revealed that the capacity for tumor propagation over several passages was associated with the presence of CD271-positive melanoma stem cells in each xenograft (Fig. 4A-C). CD271-negative cells, however, were unable to produce CD271-positive cells, even after xenograft passaging (Fig. 4A and D). It should

be noted, however, that although CD271-positive cells generate tumors in NSG mice that can be serially passaged, those tumors did not phenocopy the parental tumor (Fig. 2).

Thus, NSG mice, NK-depleted NOD/SCID mice, or NK-depleted Nude mice provided a host environment permissive for tumor growth by CD271-negative cells, whereas such cells were unable to initiate tumorigenesis in NOD/SCID or Nude mice. In contrast, CD271-positive melanoma stem cells were tumorigenic in all mouse models tested. In any case, only in Nude or NOD/SCID mice CD271-positive melanoma stem cells produced tumors that completely phenocopied the parental melanoma even upon serial xenotransplantation, while they failed to do so in NSG mice.

Expression of the NCSC markers CD271/Sox10 correlates with metastatic potential and poor prognosis of melanoma

To verify our findings with a large number of patient samples, we stained tissue microarrays containing more than 200 different melanoma biopsies of primary melanomas, melanoma metastases, and melanoma cell lines for cells co-expressing the NCSC transcription factor Sox10 (20) and CD271 that is found both in NCSCs (16, 19) and in melanoma-initiating cells (9). Consistent with its expression in NCSCs and melanocytes, nuclear Sox10 was found in the majority of cells in melanoma cell lines, primary melanomas, and metastases (Fig. 5A). In contrast, the number of cells with detectable CD271 expression was highly variable. Among primary melanoma, we found 31 biopsies without detectable CD271 expression (57%), 14 biopsies with less than 5% CD271-positive cells (26%), and 9 biopsies with more than 5% of CD271-positive cells (17%). Intriguingly, however, the proportion of CD271/Sox10-double positive cells in

primary tumors without evidence of metastasis was significantly lower than in primary tumors of patients, who developed metastases during 5 years follow-up ($p = 0.01$; Fig. 5B). In addition, there was a higher proportion of CD271/Sox10-positive cells in metastases as compared to primary tumors without evidence of metastasis ($p = 0.04$, Fig. 5C). Similarly, the proportion of CD271/Sox10-positive cells was significantly increased in cell lines derived from metastases as compared to cell lines derived from primary tumors ($p = 0.01$, Fig. 5C). Thus, both in primary tumors with evidence of metastasis and in metastatic lesions, the number of CD271/Sox10-positive cells was relatively increased, suggesting that their frequency is associated with the metastatic potential in human melanoma.

To specifically address this issue, we focused our analysis on 54 primary malignant melanomas of a sentinel lymph node study for which tumor-specific survival data were available (12). Within this cohort, a frequency of CD271/Sox10-positive cells > 5% was associated with poor tumor-specific survival ($p = 0.03$; Fig. 5D). Together, these findings support the hypothesis that an elevated frequency of melanoma cells expressing NCSC markers is a prognostic factor for the development of metastasis.

Discussion

In this study we demonstrate that human melanoma contains cells that fulfill the definition of cancer stem cells, including the capacity for extensive in vivo self-renewal, maintaining long-term tumor growth, and faithfully recapitulating the cellular composition of the patient tumor, from which the cells have been derived. These melanoma stem cells share properties with normal NCSCs, the precursors of

melanocytes in the skin. Indeed, human melanoma stem cells express the NCSC markers CD271 and Sox10, and –similar to NCSCs– have the capacity to self-renew and to generate multiple cell types in vitro and in vivo. CD271-expressing melanoma cells have most recently been shown to exhibit an increased tumor-initiating capacity as compared to CD271-negative cells in fully immunocompromised mice (9). In addition, as we show here, CD271-positive melanoma cells are not only able to initiate tumorigenesis, but invariably regenerate heterogeneous tumors analogous to the parental tumors in patients, even after serial re-transplantation. Importantly, CD271-positive cells appear to be required for continuous melanoma growth, as long-term passaging and expansion of tumors was dependent on the presence of a CD271-positive cell fraction in the tumor.

The recent debate on whether or not particular surface markers can be used to distinguish tumorigenic from non-tumorigenic melanoma cells in human biopsies (8-11) has pointed out the importance of establishing appropriate methods for cancer cell isolation from solid tumors and for the study of tumorigenic properties in vivo (3, 11, 22). Our work demonstrates that an excess of proteolytic trypsin activity during tumor digestion and cell fraction preparation can significantly reduce the percentage of cells positive for a given surface marker in immunolabelling assays. In particular, trypsin treatment substantially lowered the detectability of CD271- and ABCB5-positive cells by specific antibodies as compared to more gentle protocols of tumor digestion, presumably due to proteolytic cleavage of surface epitopes. Our findings conceivably explain the discrepancies between conflicting reports on the nature of melanoma subpopulations with tumor-initiation potential: Specific markers defining tumorigenic cells have been identified in studies avoiding trypsin during melanoma cell fraction preparation (8, 9);

our study), while no such markers were identified when trypsin has been included in the tumor digestion protocol (10, 11). Thus, we propose that trypsin treatment of melanoma cells can yield cell fractions false-negative for a given cell surface marker. The resulting contamination of supposedly marker-negative cell fractions by cells actually expressing the marker might explain why in some experimental setups cells positive for CD271 or ABCB5 as well as cells seemingly negative for these markers appeared to be equally tumorigenic and to give rise to tumors re-expressing the markers (10, 11). Moreover, loss of surface epitopes might generally affect the capacity of a tumor cell to associate with the surrounding tissue upon transplantation and to initiate tumor formation.

Strikingly, complete phenocopies of parental tumors were never obtained in NSG mice, irrespective of whether bulk tumor cells or isolated cell fractions were used for xenograft experiments. Furthermore, only fully immunocompromised NSG mice provided a host environment permissive for tumor growth by CD271-negative cells, while these cells were unable to initiate tumorigenesis in more immunocompetent models. In contrast, CD271-expressing melanoma stem cells were tumorigenic in all mouse models tested, indicating a specific capacity for immune evasion by the stem cell population. The apparent immunoselection of melanoma stem cells in xenografts involves the innate immune system, as NK cell depletion in Nude or NOD/SCID mice restored the capacity of CD271-negative cells to form tumors. In agreement with these results, NK and NKT cells have been shown to play a central part during immune surveillance of chemically induced skin tumor in mice (23-25). In addition, there are many lines of evidence supporting the concept of immunoediting also in human tumors, including melanoma (26, 27).

There are a number of mechanisms by which tumor cells may escape or suppress an immune response (28). Melanoma cells might achieve immunogenic tolerance by promoting apoptotic cell death or inactivation of antigen-reactive cells (29) or by inducing an immunosuppressive environment as provided, for instance, by increased levels of immunosuppressive macrophages and neutrophils (30). Interestingly, it has recently been shown that ABCB5-positive melanoma cells have the capacity to inhibit IL-2–dependent T-cell activation and to induce tolerization by regulatory T cells (31). Reduced expression levels of specific tumor antigens –such as Melan-A/MART-1, tyrosinase, and gp-100another mechanism for immune evasion by melanoma cells. Notably, CD271- as well ABCB5-positive melanoma cells express low levels of melanoma-associated antigens such as MART-1, supporting the idea that melanoma cells expressing melanoma stem cell markers escape the immune system attack by the host (9, 29, 31)(G.Civenni and L.Sommer, unpublished data). Of note, we identified a subpopulation of CD271-positive melanoma cells that also express ABCB5 (G. Civenni and L. Sommer, unpublished data), although the functional implication of this finding remains to be addressed. All together, these results suggest that in patients, melanoma stem cells might be able to evade or modulate the immune response, allowing these cells to promote tumorigenic growth and to provide resistance to immunotherapy. The hypothesis that these processes are relevant in patients is further supported by our data that only in mice with a certain level of immunocompetence, CD271-positive melanoma cells generated tumors fully phenocopying the original patient melanoma. However, since xenotransplantation models cannot accurately recapitulate the immune response induced by cancer in human patients, the interaction between cancer cell populations and

the immune system should be addressed using syngeneic melanoma mouse models, in which components of the anticancer immune system can be manipulated during tumor initiation and progression.

Our data are consistent with the idea that CD271-positive melanoma cells play a crucial role in tumor formation in human patients. This is further supported by our clinical data obtained with an extensive tissue array of melanoma samples. These demonstrate an association between the proportion of CD271/Sox10 positive cells in primary melanoma and metastatic disease as well as poor tumor-specific survival. Hence, high numbers of melanoma stem cells expressing NCSC markers might influence aggressiveness and the metastatic behaviour of malignant melanoma. This conceivably reflects intrinsic, NCSC-like features of melanoma stem cells, given that normal NCSCs have the capacity to extensively migrate through embryonic tissue before differentiation into melanocytes and other cell types. In support of this, increased levels of CD271 expression in melanoma has been associated with enhanced invasive potential in culture (32). Interestingly, CD271/Sox10 expression in metastatic primary melanoma was higher compared to metastatic lesions, which could be explained by different patient groups. Whereas all primary melanomas (metastatic versus non-metastatic) were primary tumors without chemotherapy or vaccination therapy, tissue of the metastases was obtained from patients after adjuvant therapies. Possibly, this therapy might result in a lower prevalence of cells with CD271/Sox10 expression in the metastases. Alternatively, the site of primary metastatic tumor formation might offer a microenvironment more favorable for stem cells than provided by distant metastases. In any case, however, the proportion of CD271/Sox10-positive cells can potentially be used as a predictor of metastases and

consequently poor tumor-specific survival. This in turn suggests that monitoring melanoma cells with NCSC-like features in primary melanoma might be of great prognostic relevance.

Several biopsies of primary, mostly non-metastatic melanoma did not contain CD271/Sox10-positive cells according to our tissue microarray analysis. CD271/Sox10-positive cells might therefore be involved in the formation of metastatic melanoma, while less aggressive tumors might originate from other types of melanoma-initiating cells. By extrapolation one could argue that tumor aggressiveness depends on whether the key oncogenic mutation occurred in a normal stem cell or in a more restricted progenitor cell (33). However, although the melanoma stem cells identified in the present study display similar marker expression and potential as normal NCSCs in the adult skin (16, 34), melanoma-initiating cells could also arise by de-differentiation of more mature melanocytic cells (35). Similarly, we cannot exclude that depending on the context or the oncogenotype, CD271/Sox10-melanoma stem cells emerge from other tumor cells, for instance upon a process reminiscent of an epithelial-to-mesenchymal transition during metastasis or by epigenetic modification (36, 37). Intriguingly, however, at least in the conditions chosen for this study, CD271 expression was a stable trait of human melanoma-initiating cells, and we did not find any evidence for CD271-positive cells originating from the CD271-negative tumor cell fraction even after prolonged incubation in vivo. Moreover, CD271-negative cells did not acquire properties of actual melanoma stem cells, in that they could neither reproduce the cellular heterogeneity of parental tumors nor sustain long-term tumor formation.

In conclusion, the identification of human melanoma stem cells required for continuous tumor growth points to potential culprits of tumor formation in patients. In the future, it might be possible to establish specific treatments that reduce tumorigenesis by elimination of these cells or by targeting 'stemness' in melanoma (1). Thus, efforts should be made to develop drugs able to promote differentiation of melanoma stem cells, to selectively kill these cells, or to specifically block their self-renewal and expansion.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Table 1

Phenocopy of the parental tumors by grafted bulk melanoma cells

Marker	% of xenografts phenocopying patient tumors (n)			
	Nude	N/S	NSG	N/S_GM1
CD271/Sox10	100% (3)	100% (7)	71% (7)	100% (4)
S100P	100% (3)	100% (7)	71% (7)	80% (4)
HMB45	100% (3)	100% (7)	100% (7)	20% (4)
MITF	100% (3)	100% (7)	57% (7)	20% (4)
Tuj1	100% (3)	100% (7)	86% (7)	60% (4)
NF	100% (3)	100% (7)	86% (7)	60% (4)
NSE	100% (3)	100% (7)	71% (7)	80% (4)
Peripherin	100% (3)	100% (7)	57% (7)	40% (4)
All markers	**100% (3)**	**100% (7)**	**0% (7)**	**0% (4)**

Capacity of unsorted melanoma cells to generate phenocopies of parental tumors in different mouse hosts. Xenografts were generated in Nude, N/S (NOD/SCID), NSG, and NOD/SCID mice treated with anti-asialo GM1 antibodies. Values represent the percentage of xenografts that phenocopy the parental tumor with respect to expression of the indicated marker. (n), number of patient samples analyzed.

Table 2

Phenocopy of the parental tumors by CD271+ melanoma cells

Marker	% of xenografts phenocopying patient tumors (n)		
	Nude CD271+	NSG CD271+	NSG CD271-
CD271/Sox10	100% (4)	100% (2)	0% (3)
S100P	100% (4)	0% (2)	66% (3)
HMB45	100% (4)	100% (2)	100% (3)
MITF	100% (4)	0% (2)	0% (3)
Tuj1	100% (4)	0% (2)	0% (3)
NF	100% (4)	100% (2)	100% (3)
NSE	100% (4)	100% (2)	100% (3)
Peripherin	100% (4)	100% (2)	33% (3)
All markers	**100% (4)**	**100% (2)**	**0% (3)**

Capacity of CD271-positive vs. CD271-negative melanoma cell fractions to generate phenocopies of parental tumors in Nude or NSG Mice. Values represent the percentage of xenografts generated by CD271-positive (CD271+) or CD271-negative (CD271-) cells that phenocopy the parental tumor with respect to expression of the indicated marker. (n), number of patient samples analyzed.

Figure Legends

Figure 1. Xenografted melanoma phenocopy the parental tumor in NOD/SCID mice, but not in NSG or NK-depleted NOD/SCID mice. (A) Immunofluorescent staining of two original human melanoma tissues (patients 31651 and 3298) and corresponding xenografts in NOD/SCID (N/S), NSG and NK-depleted NOD/SCID (N/S_GM1) mice for the NCSC-specific markers CD271 (red) and Sox10 (green) (white arrows, inserts) and the differentiation markers MelanA, S100P, HMB45, MITF, TuJ1, NF, NSE, Peripherin, and SMA (in green). Tissues were counterstained with Hoechst 33342. Expression or absence of a given marker is labelled by + or -, respectively. The lower panels show the same tissues after staining with H&E. Scale bar, 100 µm. (B) Incidence of 11 tested markers in 19 independent human metastatic melanoma lesions. (C) Frequency of cells expressing CD271/Sox10, S100P and MITF in two human metastatic melanoma tissues (patients 31651 and 3298) and in corresponding xenografts in NOD/SCID (N/S), NSG and NK-depleted NOD/SCID (N/S_GM1) mice. Values are presented as mean ± standard deviation (SD).

Figure 2. Xenografted CD271-positive melanoma cells phenocopy the parental tumor in Nude mice, but not in NSG mice. (A) The efficiency of tumor formation for independently xenografted CD271-positive or CD271-negative melanoma cells in Nude, NOD/SCID (N/S), NSG and NK-depleted NOD/SCID (N/S_GM1) mice. The numbers represent the number of growing xenografts, the values in brackets represent the total number of transplantations / the number of independent patient samples. (B) Immunofluorescent staining of a representative original human melanoma tissue (patient

2481) and corresponding xenografts after injection of CD271-positive or CD271-negative in Nude or NSG mice for the NCSC-specific markers CD271 (red) and Sox10 (green) (white arrows, inserts) and the differentiation markers MelanA, S100P, HMB45, MITF, TuJ1, NF, NSE, Peripherin, and SMA (in green). Tissues were counterstained with Hoechst 33342. Expression or absence of a given marker is labelled by + or -, respectively. Scale bar, 100 µm. (C) Melanoma cells from a xenograft (Nude) of patient sample 2481 were infected with a GFP-expressing lentivirus, and FACS-sorted $GFP^+/CD271^+$ or $GFP^+/CD271^-$ cells were plated at clonal density. Plating efficiency was 56 ± 6% for CD271-positive and 69 ± 7% for CD271-negative cells. Clones generated by CD271+ (white bars) and CD271- (black bars) cells were evaluated after 2 weeks of culture under adherent culture conditions. Values are presented as mean ± standard deviation (SD). (D) A clone derived from $GFP^+/CD271^+$ cell was cultured, revealing the emergence of multiple cells types, such as cells expressing CD271/Sox10, neuronal and melanocytic markers, and smooth muscle actin (SMA) (Fig. 3D, left panels, scale bar 100 µm). In addition, part of the clone was xenografted into Nude and NSG mice, and tumors were stained for GFP (green) and the markers described above (red) (middle and right panels, scale bar, 50 µm). Data from one representative clone out of three are shown.

Figure 3. CD271-positive melanoma cells have the capacity of self-renewal in vitro and in vivo, in contrast to CD271-negative melanoma cells. (A) Light microscopy (left panel) of a representative human melanoma sphere and immunofluorescent staining (right panel) for CD271 (red) and Sox10 (green) (white arrow, insert). Scale bar, 50 µm. (B) Propagation of $CD271^+$-derived (white bars) and $CD271^-$-derived (black bars)

spheres by serial passaging. Spheres were obtained from melanoma cell lines (M990115 and M010817 (17)), and metastatic melanoma lesions (824 and 4286). The data (mean ± SD, n = 4) are given as ratios of $2^{nd}/1^{st}$ passage and $5^{th}/4^{th}$ passage, respectively. (C) Number of CD271-positive cells (mean ± SD) in primary xenografts obtained by direct transplantation of patients' tumor material (n = 4), in secondary tumors derived from CD271-positive cells selected from primary xenografts (n = 4) (first passage), and in tertiary tumors derived from CD271-positive cells selected from secondary tumors (n = 4) (second passage). (D) Human melanoma (patient 4286) and corresponding primary and secondary xenografts derived in Nude mice from CD271-positive cell fractions were stained for the NCSC markers CD271 (red) and Sox10 (green) (white arrows, inserts) and the differentiation markers MelanA, S100P, HMB45, MITF, TuJ1, NF, NSE, Peripherin, and SMA (in green). Tissues were counterstained with Hoechst 33342. Expression or absence of a given marker is labelled by + or -, respectively. Tissues were stained with H&E (lower panels). Scale bar, 100 μm.

Figure 4. Capacity of CD271-positive cells to sustain long-term tumor growth. (A) Sorted cells from two melanoma biopsies (patients 2481 and 10477) were used to generate tumors (passage P1). From passage 2 (P2) to passage 5 (P5), cells were injected into mice as whole-tumor single-cell suspensions. 1000 cells from each fraction were injected subcutaneously. The numbers represent the number of growing xenografts, the values in brackets represent the total number of transplantations / the number of independent patient samples. CD271 expression levels were measured by FACS and are expressed as mean ± SD (n = 4). (B and C) Representative flow cytometry analysis of human melanoma xenografts (patient 2481) generated by CD271-positive cells (passage

P1) and by bulk cells (passage P2 and P3) in Nude (B) or NSG (C) mice. (D) Representative flow cytometry analysis of human melanoma xenografts (patient 2481) generated by CD271-negative cells (passage P1) and by bulk cells (passage P2 and P3) in NSG mice. CD271 signal was plotted against the side scatter (SSC-H).

Figure 5. The frequency of cells expressing the NCSC markers CD271 and Sox10 in human melanoma correlates with metastatic potential and worse prognosis. (A) Representative staining of a melanoma cell line, a primary melanoma and a metastasis for CD271 (red) and Sox10 (brown) (arrows, inserts). Scale bar, 50 μm. (B) Staining of 54 primary melanomas with known clinical course (32 developed metastases within 5 y, 22 did not) for CD271 and Sox10. The frequency of cells co-expressing CD271 and Sox10 is presented as mean ± standard deviation (SD) and median with interquartile range (IQR). (C) Staining of 32 primary and 141 metastatic melanomas as well as 22 cell lines from primary and 26 from metastatic melanoma lesions for CD271 and Sox10. The frequency of cells co-expressing CD271 and Sox10 is presented as mean ± standard deviation (SD). (D) Correlation between tumor-specific survival of melanoma patients with the frequency of CD271/Sox10-positive melanoma cells.

Figure 1

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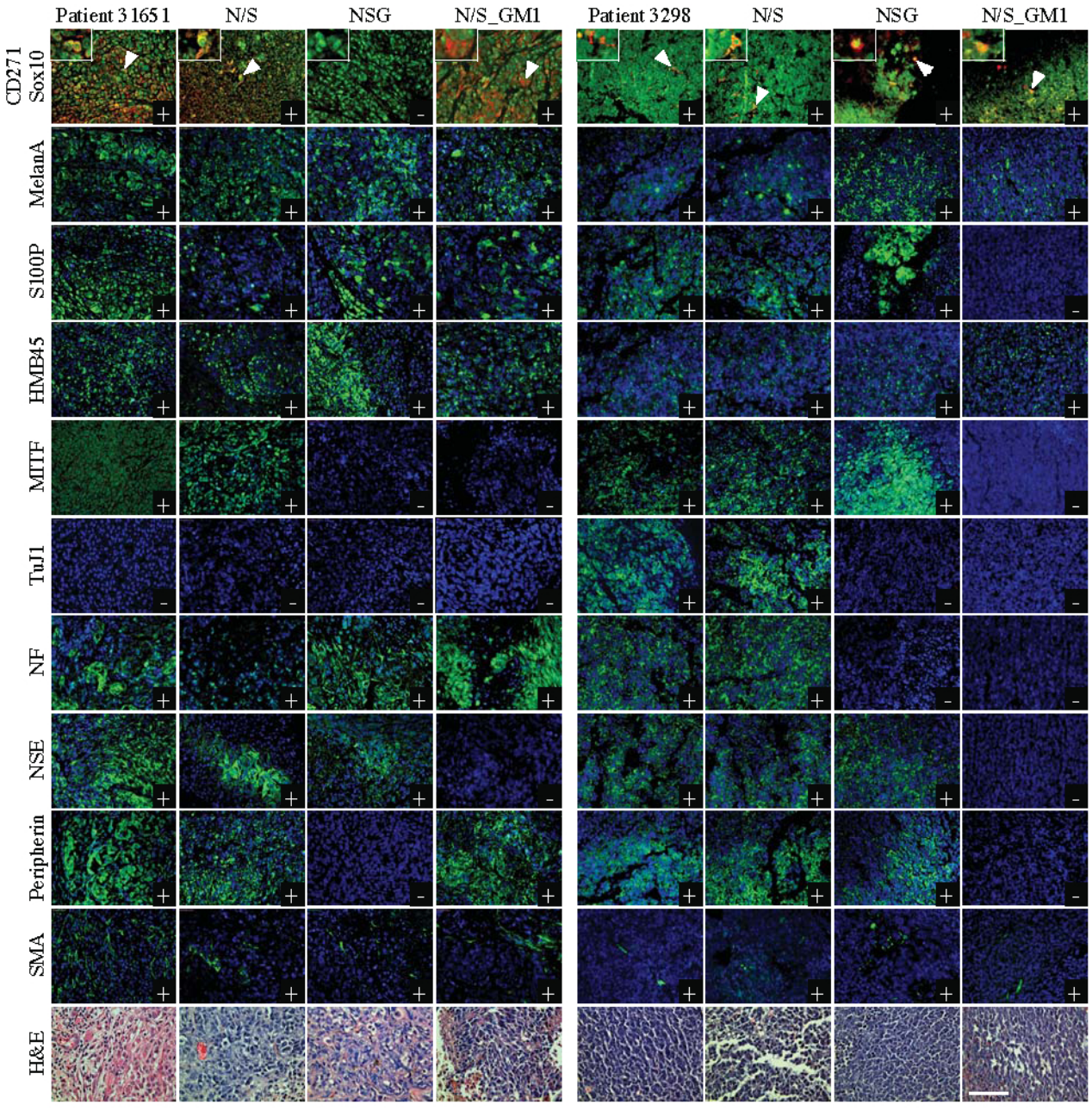
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

A Marker expression in human biopsies and xenografts



B Marker expression rate in human melanoma biopsies

Marker	Frequency
CD271/Sox10	19/19
MelanA	16/19
S100P	19/19
HMB45	15/19
MITF	17/19
TuJ1	15/19
NF	15/19
NSE	15/19
Peripherin	14/19
SMA	19/19

C Quantification of marker expression in human biopsies and xenografts

Marker		% of cells positive for given marker (± SD)			
	Patient ID	Patient	N/S	NSG	N/S_GM1
CD271/Sox10	31651	15 ± 2	10 ± 3	0	30 ± 5
	3298	8 ± 4	10 ± 2	12 ± 1	9 ± 5
S100P	31651	86 ± 7	20 ± 4	42 ± 5	53 ± 5
	3298	65 ± 10	72 ± 12	50 ± 13	0
MITF	31651	91 ± 11	83 ± 5	0	0
	3298	89 ± 5	79 ± 5	36 ± 8	0

Figure 2

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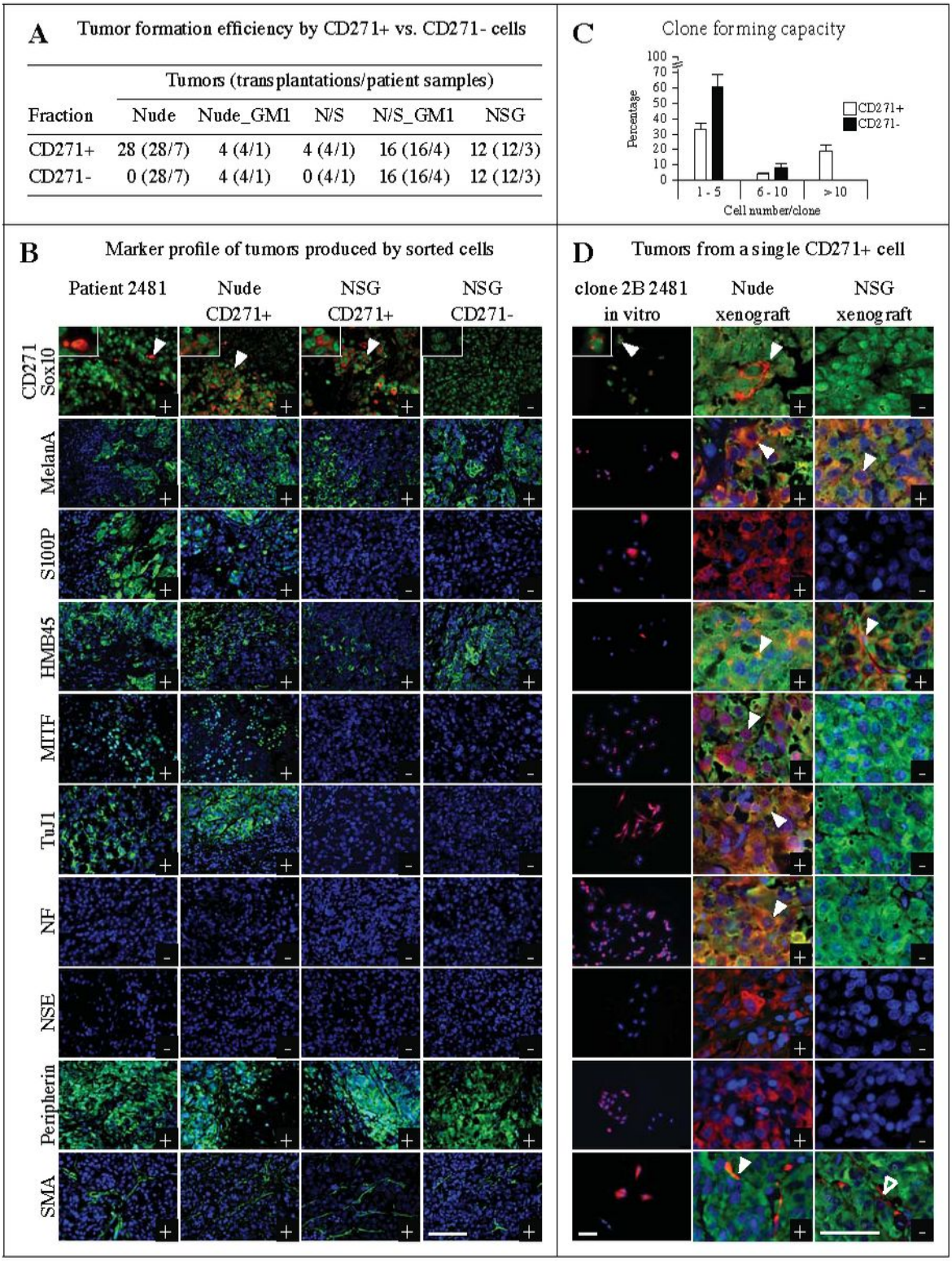

Figure 3

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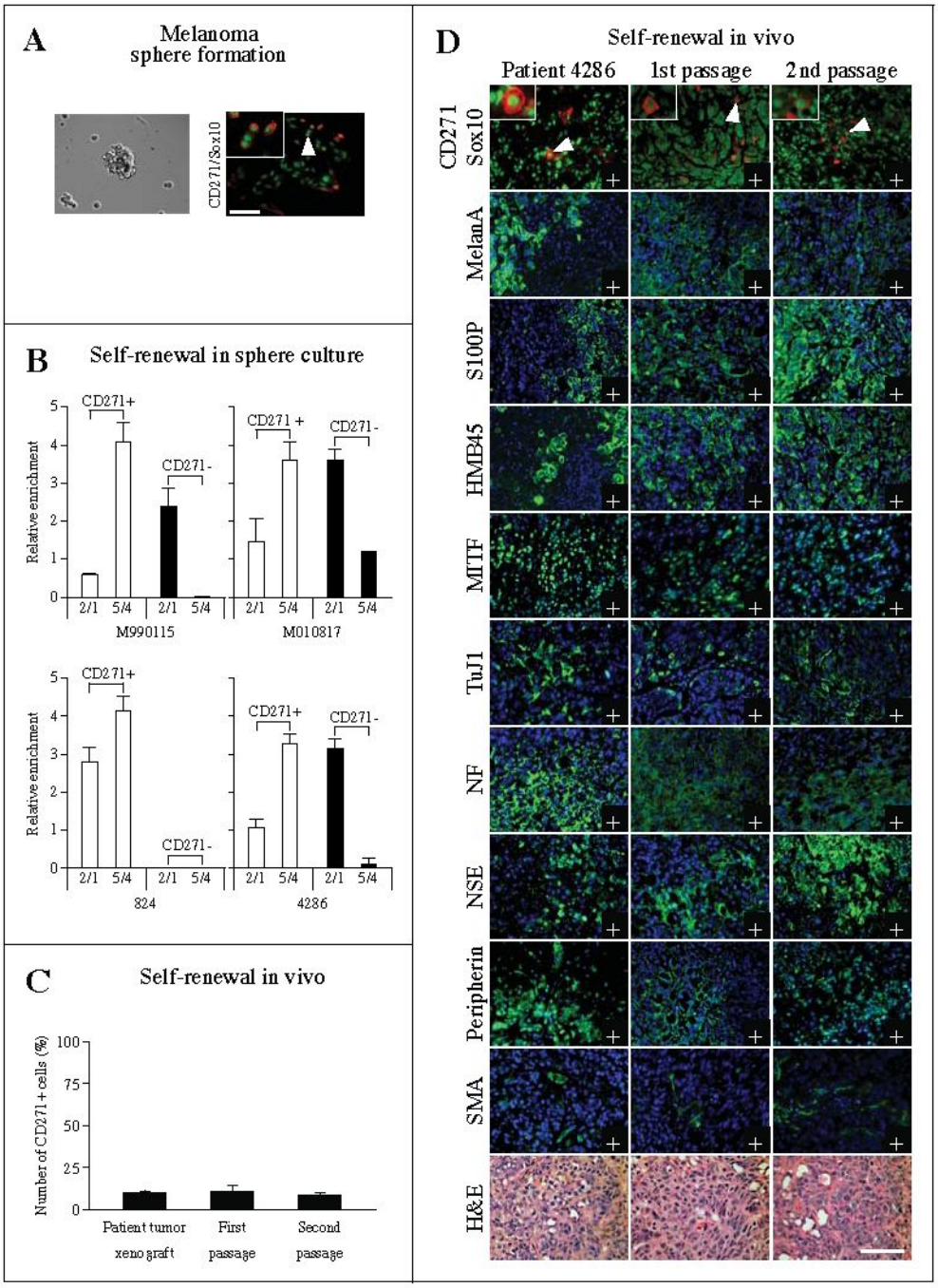

Figure 4

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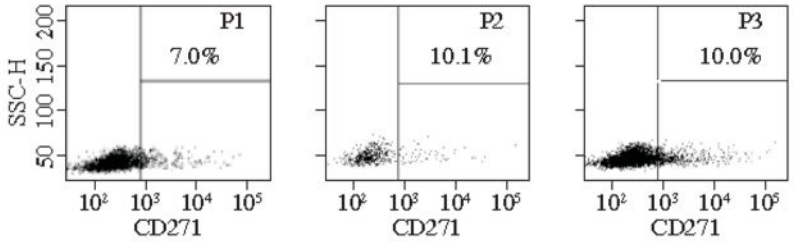
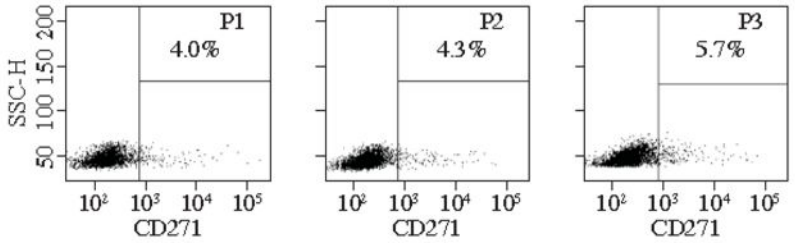
A Long-term growth of tumors containing CD271-positive cells

Mouse strain	CD271 fraction	P1 Tumor formation	P1 CD271 % ± SD	P2 Tumor formation	P2 CD271 % ± SD	P3 Tumor formation	P3 CD271 % ± SD	P4 Tumor formation	P4 CD271 % ± SD	P5 Tumor formation	P5 CD271 % ± SD
Nude	+	8(8/2)	7.0 ± 2.8	8(8/2)	10.4 ± 2.7	8(8/2)	10.1 ± 2.8	8(8/2)	6.4 ± 1.5	8(8/2)	7.5 ± 1.4
	-	0(8/2)									
NSG	+	4(4/1)	4.1 ± 1.0	4(4/1)	4.6 ± 0.7	4(4/1)	6.6 ± 0.8	4(4/1)	5.9 ± 1.8	4(4/1)	5.9 ± 3.2
	-	8(8/2)	0	8(8/2)	0	4(8/2)	0	0(4/2)			

B % of CD271-positive cells in Nude mouse xenografts; tumor derived from CD271-positive cells



C % of CD271-positive cells in NSG mouse xenografts; tumor derived from CD271-positive cells



D % of CD271-positive cells in NSG mouse xenografts; tumor derived from CD271-negative cells

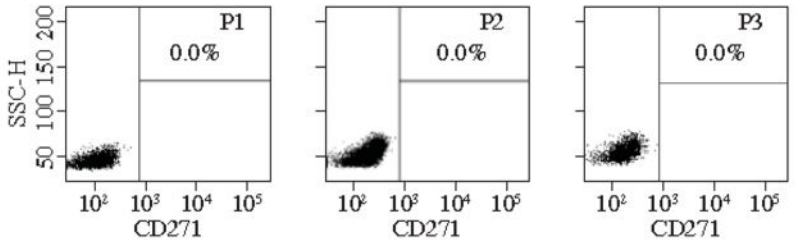


Figure 5

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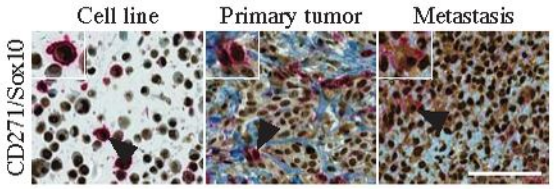
A Melanoma tissue microarrays



B CD271/Sox10 co-expression & metastatic behaviour

Primary melanoma stage	n	%CD271/Sox10 (± SD)	median [IQR]
Non-metastatic	32	4 ± 17	0 [0-1]
Metastatic	22	18 ± 35	1 [0-9.5]
p-Value		0.01	

C CD271/Sox10 co-expression in primary vs. metastatic melanoma

Stage	% CD271/Sox10 (± SD)			
	n	Tumor tissue	n	Cell lines
Primary melanoma	32	4 ± 17	22	6 ± 21
Melanoma metastasis	141	6 ± 18	26	13 ± 30
p-Value		0.04		0.01

D Tumor-specific survival